2003

# Scale-Up of the Fermentation and Purification of the Recombinant Heavy Chain Fragment C Of Botulinum Neurotoxin Serotype F, Expressed in *Pichia pastoris*

Scott K. Johnson
*University of Nebraska - Lincoln*, sjohnson8@unl.edu

Wenhui Zhang
*University of Nebraska - Lincoln*

Leonard A. Smith
*Division of Toxinology and Aerobiology, United States Army Medical, Research Institute of Infectious Diseases, Fort Detrick, Fredrick, MD*

Karen J. Hywood-Potter
*University of Nebraska - Lincoln*

S. Todd Swanson
*University of Nebraska - Lincoln*

*See next page for additional authors*

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Protein Expression and Purification 32 (2003) 1–9
Protein Expression & Purification
www.elsevier.com/locate/yprep

# Scale-up of the fermentation and purification of the recombinant heavy chain fragment C of botulinum neurotoxin serotype F, expressed in *Pichia pastoris*

Scott K. Johnson,[a] Wenhui Zhang,[a] Leonard A. Smith,[b] Karen J. Hywood-Potter,[a] S. Todd Swanson,[a] Vicki L. Schlegel,[c] and Michael M. Meagher[a,d,*]

[a] *Biological Process and Development Facility, University of Nebraska-Lincoln, Lincoln, NE 68583-0919, USA*
[b] *Division of Toxinology and Aerobiology, United States Army Medical, Research Institute of Infectious Diseases, Fort Detrick, Fredrick, MD 21702-5011, USA*
[c] *Department of Food Science and Technology, University of Nebraska-Lincoln, Lincoln, NE 68583-0919, USA*
[d] *Department of Chemical Engineering, University of Nebraska-Lincoln, Lincoln, NE 68588-0643, USA*

Received 9 October 2002, and in revised form 22 July 2003

**Abstract**

A recombinant heavy chain fragment C of botulinum neurotoxin serotype F (BoNTF(Hc)) has been expressed in *Pichia pastoris* for use as an antigen in a proposed human vaccine. *P. pastoris* cells were grown using glycerol batch, glycerol fed-batch, and methanol fed-batch methods to achieve high cell densities. The total cellular protein recovered after homogenization was 72 mg/g of cell paste. BoNTF(Hc) was purified from soluble *Pichia* cell lysate employing ion-exchange chromatographic (IEC) and hydrophobic interaction chromatographic (HIC) methods developed at the bench scale using 10–100 mL columns. The process was performed at the pilot scale using 1–4 L columns for evaluation of scale up. The purification process resulted in greater than 98% pure product consisting of at least three forms of BoNTF(Hc) based on mass spectrometry and yielded up to 205 mg/kg cells at the bench scale and 170 mg/kg cells at the pilot scale. Full-length BoNTF(Hc) is present based on mass spectrometry and SDS–PAGE, however is postulated to be N-terminally blocked by acetylation. N-terminal sequencing showed that two of the three forms are missing the first 11 (80%) and 14 (20%) amino acids of the N-terminus from the full-length form. The ratios of the two clipped forms were consistent from the bench to pilot scales. Purified BoNTF(Hc) at the pilot scale was found to sufficiently protect mice against a high dose of BoNTF neurotoxin.


There are seven serologically distinct forms (A–G) of botulinum neurotoxins (BoNT)[1] produced by *Clostridium botulinum*, each of which are the most toxic substances known to man [1]. Exposure to these agents results in the disease botulism, which leads to fatal paralysis of respiratory muscles [2]. BoNT act on nerve cells by inhibiting their release of the neurotransmitter acetylcholine at neuromuscular junctions, thus preventing muscle activation, leading to flaccid paralysis [3,5].

Active BoNT are produced by post-translational modification of a 150-kDa precursor, resulting in a di-chain consisting of a 100-kDa heavy chain and a 50 kDa light chain connected by a disulfide bond [5,6]. The C-terminus of the heavy chain (Hc) participates in the

* Corresponding author. Fax: 1-402-472-4985.
*E-mail address:* mmeagher1@unl.edu (M.M. Meagher).

[1] *Abbreviations used:* BCA, bicinchoninic acid; BoNT, botulinum neurotoxin; BoNTF(Hc), botulinum neurotoxin serotype F, heavy chain C-terminal fragment; BMGY, buffered minimal glycerol complex medium; BSA, bovine serum albumin; BSM, basal salts media; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CV, column volume; DO, dissolved oxygen; EDTA, ethylenediaminetetraacetic acid, disodium; ELISA, enzyme-linked immunosorbent assay; FF, fast flow; Hc, heavy chain C-terminal fragment; HIC, hydrophobic interaction chromatography; HPLC, high performance liquid chromatography; IEC, ion-exchange chromatography; kDa, kilodalton; MW, molecular weight; PEI, polyethyleneimine; PTM1, *Pichia* trace minerals 1; PVDF, polyvinylidene difluoride; Q, quaternary amine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SP, sulfopropyl; WCW, wet cell weight.

1046-5928/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2003.07.003

binding to specific receptors on cholinergic nerve cells [7–12]. The N-terminal portion of the heavy chain functions in translocation of the light chain of the neurotoxin across the endosomal membrane [13–16]. The light chain, a zinc-endopeptidase [4,17–19], then proteolytically cleaves key synaptic vesicle proteins, which are required for neurotransmitter release [4,17–20]. For nerve intoxication to completely occur, all portions of the BoNT must be present. The Hc fragments of BoNT A, B, C, E, and F were shown to be nontoxic, antigenic [21], and capable of eliciting a protective immunity in animals challenged with homologous BoNT [22,23]. Therefore, these Hc fragments are being developed as vaccine candidates for prevention of botulism [24–29].

A fermentation protocol utilizing glycerol batch, glycerol fed-batch, and methanol fed-batch phases allowed high cell densities to be reached. The induction time was kept short (10 h) to optimize BoNTF(Hc) production while minimizing the proteolytic degradation. Initial runs performed at the bench scale resulted in much lower product yields than expected, which led us to optimize the cell breakage step. A total of three bench-scale runs were performed using cells generated from either a 60 or 5 L fermentation. Two pilot-scale purification runs were performed using cells generated from the same 60 L fermentation to determine the ability of this method to be scaled-up. For purification processes, bench scale refers to purifications performed using columns less than 150 mL, while pilot scale refers to processing with columns on the $> 0.5$ L scale (see Materials and methods for actual column volumes).

## Materials and methods

### *Five and 60 L fermentation*

A recombinant strain of *Pichia pastoris* (GS115) transformed with pHIL-D4 plasmid containing a synthetic gene encoding the putative heavy chain fragment of botulinum serotype F (24) was used as seed culture for all fermentations. A 1-L baffled shake flask containing 150 mL BMGY (buffered minimal glycerol complex medium, *Pichia* Expression Kit, Invitrogen, USA) was inoculated with 1 mL of frozen strain stock and incubated at 30 °C, 200 rpm until an $OD_{600}$ (optical density at 600 nm) between 4 and 8 was reached. The entire 150 mL was employed as an inoculum for a 5-L fermentor (Bioflo 3000, New Brunswick Scientific, USA) containing 3 L of BSM plus 0.435% v/v PTM1. BSM consists of (per liter) 26.7 mL of 85% $H_3PO_4$, 0.93 g $CaSO_4$, 18.2 g $K_2SO_4$, 14.9 g $MgSO_4 \cdot 7H_2O$, 4.13 g KOH, and 40.0 g glycerol; and PTM1 consists of (per liter) 6.0 g $CuSO_4 \cdot 5H_2O$, 0.08 g NaI, 3.0 g $MnSO_4 \cdot H_2O$, 0.2 g $Na_2MoO_4 \cdot 2H_2O$, 0.02 g $H_3BO_3$, 0.5 g $CoCl_2$, 20.0 g $ZnCl_2$, 65.0 g $FeSO_4 \cdot 7H_2O$, 0.2 g biotin, and 5.0 mL $H_2SO_4$. Prior to inoculation, the pH was adjusted to 5.0 with concentrated ammonium hydroxide. The fermentation was controlled at pH 5.0, temperature 30 °C and dissolved oxygen (D.O.) >20% of saturation. When the fermentation reached a density of 30–50 g/L wet cell weight (WCW), the culture was transferred to a 80-L fermentor (MPP 80, New Brunswick Scientific) containing 40 L of BSM plus 0.435% PTM1. When glycerol was exhausted, which was indicated by a D.O. spike, a glycerol feed was initiated and lasted for 4 h. The feed rate has the following profile: first hour, 20 g/h/L (g 50% w/v glycerol containing 1.2% v/v PTM1/h/L initial medium); second–fourth hour, feed rate decreased linearly from 20 g/h/l to 0. At the end of first hour, methanol was added to the fermentation vessel to achieve a concentration of 1.5 g/L. At the end of the third hour, a methanol feed was initiated and the feed rate was programmed to increase linearly using the following profile: 0 h, 4 g/h/L (g 100% methanol containing 1.2% v/v PTM1/h/L initial medium); 2.4 h, 6 g/h/L; 3.8 h, 7 g/h/L; and 8.5 h, 9 g/h/L. The methanol feed rate was adjusted using the dissolved oxygen spike method [30]. The total induction time on methanol was 10.5 h. The final cell density was about 170 g/L wet weight.

### *5 L cell harvest and disruption*

Cells harvested from a 5 L fermentation were spun at 8000*g* at 4 °C for 20 min using a Beckman J2-21 Centrifuge (Palo Alto, CA). Cell paste was then either frozen at −20 °C or processed immediately. Cell paste was resuspended in 25 mM sodium acetate + 5 mM EDTA, pH 5.0, to either 10 or 26% (w/v) solids. Cells were then homogenized using a Microfluidizer M-110EH (Microfluidics, Newton, CA) set at 21,000 psi. A total of 3–5 passes were performed to obtain at least 75% cell disruption. The homogenate was then brought to 0.25% Polyethyleneimine (PEI) using a 5% (w/v) stock solution at pH 7.0. This was allowed to mix for 30 min at 4 °C. The resulting mixture was processed by centrifugation at 10,000*g* for 20 min at 4 °C. The supernatant was then decanted, passed through a 0.2 μm cellulose acetate filter, and saved for further immediate processing.

### *Bench scale purification*

All bench scale chromatographic separations were performed on a BioCad Workstation (PE Biosystems, Foster City, CA) at room temperature, with the load material on ice. Supernatant was passed through a 0.2 μm polyethersulfone (PES) filter and was loaded onto a 100 mL SP Sepharose FF column (2.6 × 19 cm) (Amersham Pharmacia) equilibrated with 5 column

volumes (CV) of 25 mM sodium acetate + 1 mM EDTA, pH 5.0. The linear velocity used for this column was 250 cm/h. The column was washed with 5 CV equilibration buffer, followed by a wash step with 25 mM sodium phosphate + 1 mM EDTA, pH 6.8. The product was eluted using a 20 CV linear gradient from 0 to 1 M sodium chloride in 25 mM sodium phosphate + 1 mM EDTA, pH 6.8. The product starts to elute at a conductivity of 20 mS/cm.

The SP Sepharose product was adjusted to 1 M ammonium sulfate using a 3.5 M ammonium sulfate stock. The adjusted solution was allowed to mix at room temperature for at least 30 min and then filtered through a 0.2 μm filter. The filtered material was then loaded onto a 32 mL butyl Sepharose 4 FF column (1.6 × 16 cm) (Amersham Pharmacia) equilibrated with 5 CV 1 M ammonium sulfate + 25 mM sodium phosphate + 1 mM EDTA, pH 6.8. The linear velocity used for this column was 300 cm/h. The column was washed with 5 CV of equilibration buffer and the product was eluted using a 10 CV linear gradient from 1 to 0 M ammonium sulfate in sodium phosphate + 1 mM EDTA, pH 6.8. BoNTF(Hc) elutes as the second major peak at approximately 60 mS/cm conductivity.

The butyl Sepharose product was dialyzed vs. 20 volumes of 25 mM sodium acetate + 1 mM EDTA, pH 5.0, using 10 kDa Slide-A-Lyzer cassettes (Pierce, Rockford, Il) at 4 °C. The dialyzed product was loaded onto a 13-mL ToyoPearl SP 650M column (1.0 × 17 cm) (TosoHaas, Montgomeryville, PA) equilibrated with 5 CV of 25 mM sodium acetate + 1 mM EDTA, pH 5.0. The linear velocity used for this column was 300 cm/h. After loading, the column was washed with 5 CV of equilibration buffer. BoNTF(Hc) was eluted from the column using a 20 CV linear gradient from equilibration buffer to 100 mM sodium phosphate + 1 mM EDTA, pH 7.0. The product elutes at the end of the gradient as the second major peak at approximately 13 mS/cm conductivity. Final product was then stored at −20 °C.

*60 L cell harvest and disruption*

Fermentation broth was diluted to 10% solids with distilled water prior to centrifugation and harvested using a Westfalia CSA8 disk-stack separator (Oelde, Germany). The resulting cell paste was either frozen at −20 °C or processed immediately. The cell paste was brought to 21–26% (w/v) solids using 25 mM sodium acetate + 5 mM EDTA, pH 5.0. Cells were disrupted using an APV Gaulin 30-CD Homogenizer (Everett, MA) at 16,000 psi maintained at 10 °C. Harvested cells were exposed to 3–5 passes through the homogenizer and cooled to 10 °C prior to the next pass using a chilled heat exchanger. The resulting homogenate was brought to 0.25% PEI using a 5% (w/v) stock and allowed to mix for 30 min at 10 °C. The resulting mixture was separated using the Westfalia described above. The supernatant was sterile-filtered using 10″ 0.2 μm Fluorodyne II filters (Pall, East Hills, NY) and transferred to the purification pilot plant for further processing.

*Pilot scale purification*

All chromatographic separations were performed on a NC-SRT pilot-scale chromatography skid. For each chromatographic step, conditions were the same as the bench-scale work, i.e., identical linear velocities, equilibration, washing, and elution methods. The SP Sepharose FF step was performed using a 4 L (20 × 13 cm) BPG 200/500 column (Amersham Pharmacia Biotech, Piscataway, NJ). The butyl Sepharose 4 FF step was performed on a 1.3 L (10 × 16 cm) BPG 100/500 column (Amersham Pharmacia), while the final ToyoPearl SP 650M step was performed using a 526 mL (6.1 × 18 cm) Vantage 60A column (Millipore, Bedford, MA). The SP Sepharose product was brought to 1 M ammonium sulfate by addition of granular ammonium sulfate. The diafiltration step was performed using a 3 sq. ft. 10 kDa spiral wound membrane (Millipore). The material was diafiltered with 3 volumes of 25 mM sodium acetate + 1 mM EDTA, pH 5.0, at which time the retentate pH was 5.0 and the conductivity 4.7 mS at 10 °C. The retentate was then concentrated approximately 2-fold and filtered through a 0.45 μm CA filter prior to loading onto the final column. Final product of the ToyoPearl SP 650M column was sterile-filtered through a 0.2 μm Supor capsule filter (Pall, East Hills, NY) and stored at −20 °C.

*Protein analysis*

Total protein concentrations were determined using the BCA (Pierce) Standard Assay, using BSA as a standard. Purity was determined by SDS–PAGE using 4–20% polyacrylamide gels (Novex, San Diego, CA) stained with Coomassie blue or silver stain. Western blot analysis was performed using polyclonal Protein G-Sepharose-purified horse anti-BoNTF antibody incubated at 1 μg/mL for 1 h. The secondary antibody was a horseradish peroxidase labeled affinity-purified goat anti-horse IgG (Kirkegaard & Perry Laboratories, Gainsburg, MD) incubated at 1 μg/mL for 1 h. SDS–PAGE-separated proteins were transferred to PVDF membranes (BioRad, Hercules, CA) at 100 V for 1 h, blocked with 5% non-fat dried milk for 1 h, and washed with 0.05% Tween 20 in phosphate-buffered saline (PBS) prior to treatment with antibodies. Blots were then visualized by Chemiluminescence using the ELC plus Detection Kit (Amersham Pharmacia Biotech, Piscataway, NJ). N-terminal sequencing was performed

by the University of Nebraska-Medical Center Protein Core Facility using automated Edman degradation performed on a Procise model 491-HT amino acid sequencer (PE Biosystems, Foster City, CA). Liquid chromatography quadrupole time-of-flight mass spectrometry (LC Q-TOF MS) experiments were performed at the University of Nebraska Center for Mass Spectrometry using a Micromass Q-TOF mass spectrometer coupled to a Shimadzu HPLC (Shimadzu, Kyoto, Japan) using a C4 reverse phase Micro trap column (Michrom Bioresources, Auburn, CA). For LC separation, buffer A consisted of 0.1% formic acid in water and buffer B consisted of 0.1% formic acid in acetonitrile. After loading, the column was washed for 8 min at 10% buffer B. The BoNTF(Hc) was eluted from the column using a 5-min gradient from 10 to 70% buffer B. Elution was monitored by total ion current and base ion current detection using the MassLynx$^{TM}$ software (Waters, Milford, MA). Raw mass spectroscopy data were deconvoluted to generate parent ions mass spectra.

*Mouse inoculations and BoNTF toxin challenge*

The efficacy of purified BoNTF(Hc) was determined by toxin challenge studies on mice. Groups of 10 female mice (CD1, ICR mice (Charles Rivers, NC) weighing 16–22 g on receipt) were intramuscularly vaccinated (into the caudal muscle of the right rear limb) with two (2×) or three (3×) doses of either 0.04, 0.2, 1 or 5 μg BoNTF(Hc). Vaccine was diluted in 0.2% (v/v) Alhydrogel (HCI Biosector, Frederikssund, Denmark) containing 100 mM sodium chloride and injections were administered at 4 week intervals (100 μL/injection). Mice were challenged 14 days post-vaccination with 1000 mouse $LD_{50}$ with BoNTF toxin complex (Langeland strain) where the 1000 mouse $LD_{50}$ is 1000 times the dose (by volume) necessary to kill 50% of mice in a group being challenged. Mice were observed daily and deaths were recorded 5 days post challenge.

## Results and discussion

*Bench scale fermentation and purification*

Seed cultures of a recombinant strain of GS115 *P. pastoris* containing the pHIL-D4 plasmid containing a synthetic gene encoding the putative heavy chain fragment of botulinum serotype F [24] were used as an inoculum. Five-liter bench scale fermentations involved standard methanol feed rates (see Materials and methods), with up to a 10-h induction. This short induction time was necessary to minimize the effect of proteolytic cleavage, yet still reached sufficient levels of expression.

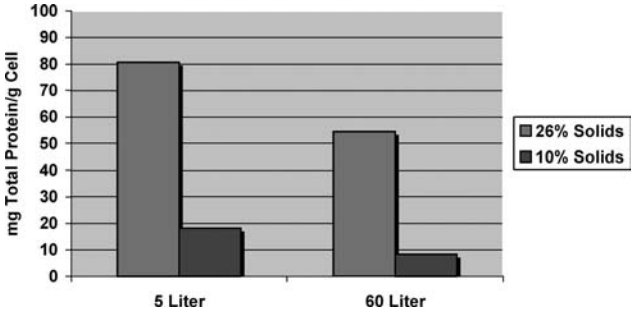



Fig. 1. Effect of percent solids (w/v) on cell breakage. Cells harvested from 5 and 60 L fermentations were adjusted to the appropriate solids (w/v), homogenized, and exposed to centrifugation. Resulting supernatants were analyzed for total protein concentration using the Bradford protein assay (BioRad) using BSA as the standard.

Initial harvest processing resulted in a significantly lower product yield than expected (54.35 mg BoNTF (Hc)/kg cell) compared to published results [24]. It was postulated that cell breakage at a higher percent solids would result in a much higher amount of cell lysis (the term "solids" is defined as the mass of cell paste present per volume of solution). An experiment was performed to test the amount of total protein released by homogenization at different percent solids (see Fig. 1). Cells were broken using a bead beater set up. This breaking system consists of cell pellet mixed to the appropriate percent solids with breaking buffer and an equal volume of Zirconia beads placed in a blending apparatus. Cells were broken by six cycles of 1 min blending and 5 min cooling on ice. These results show a 6-fold increase in the amount of protein released by increasing the percent solids from 10 to 26%. These results were also confirmed during breakage using the Microfluidizer described in Materials and methods. We also found that for optimum release of protein during disruption, harvested cell paste should be processed immediately and not frozen. It is postulated that extended frozen storage of cells causes them to be more difficult to disrupt [28]. Therefore, subsequent cell disruptions were performed at 26% solids and were conducted immediately after harvesting.

Harvested material was purified at the bench scale using ion exchange and hydrophobic interaction chromatography techniques. SP Sepharose FF resin was used to capture the target product due to its direct scalability and sufficient binding capacity for BoNTF(Hc) antigen. The maximum total protein loaded onto the SP Sepharose FF column was 55.5 mg/mL resin. At this load, less than 3% of the product introduced to the column was seen in the flow through. Material eluted from the capture column was further processed through a butyl Sepharose FF. This resin was found to remove a majority of the smaller molecular weight *Pichia* proteins present after capture. A maximum protein load of 4.8 mg/mL resin was used for the butyl Sepharose 4 FF

Table 1
Bench scale purification of BoNTF(Hc)[a]

| Step | Volume (mL) | $[Protein]_{Total}$ (mg/mL) | Total protein (mg) | Estimated purity[b] (%) | BoNTF (Hc)[c] (mg) | Step yield (%) | Total yield (%) |
|---|---|---|---|---|---|---|---|
| Lysate | 820 | 13.26 | 10873 | $<0.5$ | 54.4 | (100) | (100) |
| PEI-treatment | 740 | 7.65 | 5661 | <1 | 45.3 | 83 | 83 |
| SP Sepharose FF | 270 | 0.64 | 174 | 25 | 43.4 | 96 | 80 |
| Adjusted SP Sepharose FF | 380 | 0.42 | 161 | 26 | 41.9 | 97 | 77 |
| Butyl Sepharose 4 FF | 87 | N.D. | N.D. | 87 | N.D. | N.D. | N.D. |
| Dialyzed butyl Sepharose 4 FF | 88 | 0.43 | 37.9 | 87 | 33.0 | N.D. | 61 |
| ToyoPearl SP 650M | 65 | 0.36 | 23.3 | 98 | 22.9 | 69 | 42 |

N.D., not determined.
[a] Purification is from 150 g cells wet weight.
[b] Estimated by visual inspection of SDS–PAGE.
[c] Determined by multiplication of total protein and purity.

column and Western blot analysis of the flow through showed no signs of BoNTF(Hc) present. A ToyoPearl SP 650M resin was used as a polishing column to remove a 63 kDa contaminant as well as some smaller molecular weight proteins. A total protein load of 2.7 mg/mL resin for this final column resulted in no BoNTF(Hc) detected in load flow through. The entire purification process, from harvesting to final sterile-filtering, was performed in 20 h.

For an overall evaluation of this purification process, a total of three purification processes were performed at the bench scale. Results of these purification runs were very similar in product yield, purity, and elution profiles. Purification runs resulted in final product yields, ranging from 155 to 205 mg BoNTF(Hc)/kg cells (Table 1). A typical purification resulted in at least 98% pure BoNTF(Hc), which consisted of at least two forms of the product based on SDS–PAGE (Fig. 2). Analysis by N-terminal sequencing shows the prominent form (80% of the total) of the product to be missing the first 11 amino acids (−11) and a second form (20% of the total) missing the first 14 amino acids of the amino terminus (−14) (see Table 2). The ratio of product forms was consistent for all purification processes performed at both the bench and pilot scales. Results from these two analytical methods are conflicting due to the fact that SDS–PAGE shows the lower band (−14) to have the greatest intensity of the two, while the −14 form is only 20% of the total by N-terminal sequencing.

To shed light on this inconsistency, liquid chromatography-quadrupole time-of-flight mass spectrometry analysis was performed. We postulated that the N-terminus was acetylated which would block N-terminal sequencing of this form and that the −11 and −14 were not being resolved by SDS–PAGE. Results of the MS experiment shows three groups of species of BoNTF(Hc) present; (1) $50{,}161 \pm 5.69$ Da, (2) $48{,}959 \pm 2.93$ Da, and (3) $48{,}533 \pm 9.20$ Da (see Fig. 3). The main component of each peak group was used for data analysis. Specie 1 is consistent with removal of the

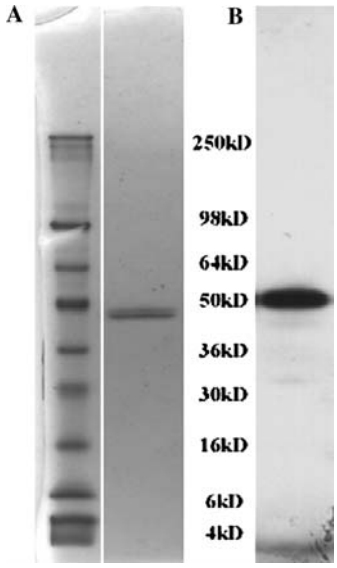
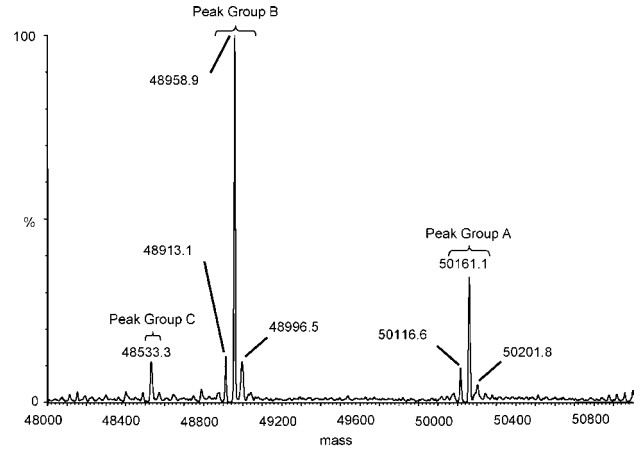



Fig. 2. (A) Silver stained SDS–PAGE and (B) chemiluminescent Western blot analysis of purified BoNTF(Hc) from a typical bench-scale purification (PDL-004). (A) Lane 1 is MW standards and lane 2 is 2.5 μg final product and (B) lane 1 is 50 ng final product.

Table 2
N-terminal sequence of purified BoNTF(Hc)

| Sample | N-Terminal sequence | |
|---|---|---|
| Intact BoNTF(Hc) | MSYTN DKILI LYFNK LYKKI KDNSI | |
| Bench scale PDL-004 | YFNK LYKKI K | 80% (−11) |
| | K LYKKI XDNX | 20% (−14) |
| PDL-005 | YFNK LYKKI K | 75% (−11) |
| | K LYKKI KDN | 25% (−14) |
| PDL-006 | YFNK LYKKI K | 76% (−11) |
| | K LYKKI XDNX | 24% (−14) |
| PDL-007 | YFNK LYKKI K | 80% (−11) |
| | K LYKKI KDN | 20% (−14) |
| Pilot scale PPP-006 | YFNK LYKKI K | 89% (−11) |
| | K LYKKI KDN | 11% (−14) |
| PPP-007 | YFNK LYKKI K | 77% (−11) |
| | K LYXXI XDNS | 23% (−14) |




| Peak Group | Relative Intensity | Normalized Intensity |
|---|---|---|
| A | 31% | 4 |
| B | 61% | 8 |
| C | 8% | 1 |

Fig. 3. Liquid chromatography quadrupole time-of-flight mass spectra of purified BoNTF(Hc). Relative intensities of each peak group are displayed along with normalized intensities for comparison. Raw mass spectroscopy data were deconvoluted to generate parent ions mass spectra. The main component of each peak group was used for data analysis.

N-terminal methionine and acetylation which has a theoretical mass of 50,172.5 Da (the theoretical full-length BoNTF(Hc) mass is 50,261.5 Da). Acetylation of N-terminal serine has been documented in yeast [30]. Species 2 and 3 are consistent with the −11 and −14 forms of BoNTF(Hc), respectively. These mass spectrometry experiments are preliminary and require more detailed experimentation to fully resolve this issue, however we feel these data provide support for our hypothesis of N-terminal acetylation of our product.

It was postulated that the two shortened BoNTF(Hc) forms may be the result of proteolytic degradation during fermentation/harvesting or incomplete cellular processing. Fermentation was performed with a shortened methanol induction time of 3 h, in an attempt to avoid prolonged exposure of product to protease activity and still produce sufficient amounts of full-length BoNTF(Hc). Immediate purification of this fermentation resulted in a 2-fold reduction of product yield and N-terminal sequence analysis showed both forms of the clipped product similar to previous purifications.

Initial bench-scale processing showed minor higher molecular weight contaminates present in the final BoNTF(Hc) product. An attempt to remove these *Pichia* proteins was performed using the zwitterionic detergent, CHAPS as an additive in the initial column buffers. We found that 0.25% CHAPS did not aid in the removal of these proteins (data not shown). Also unsuccessful was an attempt using a negative purification step with Q Sepharose FF following the capture step with CHAPS present in the buffers. BoNTF(Hc) from this experiment did not show an increase in purity. We did find that these contaminants may be avoided by making a very detailed cut in collection of product eluting from the SP Sepharose FF column. Such detail in fraction collecting is difficult to perform at the pilot scale, and leads to lower product yields as the elution of these contaminants and BoNTF(Hc) overlap.

### *Pilot scale fermentation and purification*

Two 60 L fermentations and two pilot scale purification runs were performed. These fermentations resulted in final wet cell weights of 21.2–30.8% with final volumes of 56 and 57 L, respectively.

The initial pilot scale purification run was performed using freshly harvested cells and resulted in 179 mg BoNTF(Hc)/kg cell. The product from the SP Sepharose FF capture column was collected in bulk with collection beginning as soon as the $Abs_{280}$ began to increase. Upon completion of this purification run, the final product was found to contain two minor higher molecular weight contaminants. These contaminants were the result of the "broad" cut made during product collection off the capture column. These contaminants co-elute significantly with BoNTF(Hc) and proved difficult to remove during subsequent chromatography steps.

A second pilot scale purification was performed using frozen cells from the same fermentation run and generated a higher purity product (Fig. 4), however yielded 5-fold less BoNTF(Hc)/kg cell paste (Table 3). During

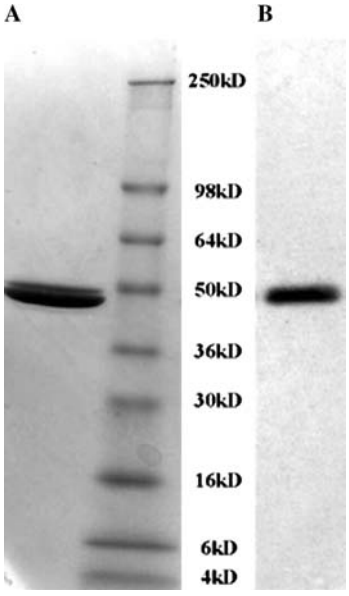



Fig. 4. (A) Coomassie blue-stained SDS–PAGE and (B) chemiluminescent Western blot of purified BoNTF(Hc) from a pilot-scale purification (PPP-007). (A) Lane 1 is 5 μg final product and lane 2 is MW standards. (B) Lane 1 is 50 ng final product.

Table 3
Pilot scale purification of BoNTF(Hc)[a]

| Step | Volume (L) | $[Protein]_{Total}$ (mg/mL) | Total protein (g) | Estimated purity[b] (%) | BoNTF (Hc)[c] (g) | Step Yield (%) | Total yield (%) |
|---|---|---|---|---|---|---|---|
| Lysate | 15 | 15.21 | 228 | <2 | 1.14 | (100) | (100) |
| PEI-Treatment | 46 | 0.80 | 36.8 | <3 | 0.92 | 81 | 81 |
| SP Sepharose FF | 15.5 | 0.11 | 1.7 | 25 | 0.43 | 47 | 38 |
| Adjusted SP Sepharose FF | 16.5 | 0.10 | 1.6 | 33 | 0.39 | 91 | 34 |
| Buytl Sepharose 4 FF | 3.5 | 0.10 | 0.4 | 80 | 0.28 | 72 | 25 |
| Diafilterd butyl Sepharose 4 FF | 2.5 | 0.07 | 0.2 | 80 | 0.13 | 47 | 12 |
| ToyoPearl SP 650M | 2.3 | 0.04 | 0.1 | 98 | 0.09 | 69 | 8 |

[a] Purification is from 2.87 kg cells wet weight.
[b] Estimated by visual inspection of SDS–PAGE.
[c] Determined by multiplication of total protein and purity.

this second purification process, product collection began only at the apex of the elution peak off the capture column. This was performed based on development data, which showed the higher molecular weight contaminants co-eluting in the front half of the product peak.

The entire purification process from harvesting to final sterile-filtering took about 25 h, similar to the time required for bench scale processing. Processing at the pilot scale resulted in a similar product yield per kilogram cell. The changes in processing relative to the bench scale were in the type of equipment used to perform certain tasks, i.e., dialysis cassettes vs. diafiltration systems. In order to remove cell debris and flocculated nucleic acid from cellular homogenate, a disk-stacked separator was used rather than conventional laboratory centrifugation. This equipment was able to reduce the harvest process time by one-half and resulted in the same quality material seen at the bench scale.

To adjust the butyl Sepharose 4 FF product to 25 mM sodium acetate + 1 mM EDTA, pH 4.5, a diafiltration system was used with a jacketed stainless-steel sample reservoir, chilled to 7 °C. This step resulted in an insignificant loss of product and took only 2 h to perform using a 10-kDa spiral-wound regenerated cellulose membrane. During adjustment of the SP Sepharose FF product to 1.0 M ammonium sulfate, some precipitation was seen initially, however, if added slowly in increments over a 1-h period with continuous mixing, this was minimized. This phenomenon was more prominent at the pilot scale, possibly due to an increased sample temperature upon mixing. During adjustment at the pilot scale, the sample was approximately 17 °C, three times the temperature this step was performed at for the bench scale process, which may have caused the protein to be less stable.

*Mouse efficacy studies*

To determine the efficacy of the purified BoNTF(Hc), mouse challenge studies were performed using purified pilot plant material using doses ranging from 0.04 to 5 μg/mouse. All mice were challenged ip with 1000 $LD_{50}$ BoNTF toxin complex. A double inoculation of 5 μg dose protected nine of 10 mice, while with a triple inoculation all 10 mice survived (Table 4). All 10 mice survived with two doses of 1 μg, whereas nine of 10 mice were protected with three doses of the same amount. When a low dose of 0.04 μg was used, seven of 10 mice survived whether they received two or three inoculations. None of the control mice survived (0/10). With a dose of 1 μg or greater, 95% (19/20) of the mice were protected using either a double or triple inoculation schedule. All 10 mice survived when inoculated three times with 0.2 μg, while eight of 10 mice survived when inoculated two times, suggesting that increased inoculations provide increased protection.

Table 4
Survival of mice after vaccination with purified BoNTF(Hc)

| Inoculation dose (μg) | Survival (alive/10 tested) | |
|---|---|---|
| | 2× | 3× |
| 5 | 9 | 10 |
| 1 | 10 | 9 |
| 0.2 | 8 | 10 |
| 0.04 | 7 | 7 |
| PBS | 0 | 0 |

## Conclusion

The production and purification of recombinant BoNTF(Hc) from *P. pastoris* using the process described above has been scaled-up and can result in a similar product purity at both the bench and pilot scales. Fermentations achieved high cell densities and reached optimum BoNTF(Hc) production after 10 h induction. Purified BoNTF(Hc) at the pilot scale was found to offer adequate protection in mouse challenge studies. It was found that to achieve maximum

BoNTF(Hc) yield, purification should be performed immediately upon completion of the fermentation. The purification process involves three chromatographic steps, which is typical of methods used to purify non-secreted products from *Pichia*, and results in three product forms, an N-terminal acetylated form missing a methionine residue and two clipped forms missing the first 11 and 14 amino acids, respectively. The ratio of clipped forms was found to be consistent regardless of processing at the bench or pilot scales. This material provided adequate protection of mice against 1000 $LD_{50}$ BoNTF toxin complex (see Materials and methods) when inoculated with three doses of 1 μg BoNTF(Hc) over a 12-week period.

## Acknowledgments

The authors of this paper thank Dr. Ron Cerny of the University of Nebraska Center for Mass Spectrometry for his expert assistance during performance in the mass spectrometry experiments. We also thank Judy Lutgen and John Regier for running the gels and total protein assays and Clem DeWitt for performing animal efficacy assays.

## References

[1] C. Lamanna, E.R. Hart, Relationship of lethal toxic dose to body weight of the mouse, Toxicol. Appl. Pharmacol. 13 (1968) 307–315.

[2] J.H. Anderson, G.E. Lewis, Clinical evaluation of botulinum toxoids, in: G.E. Lewis (Ed.), Biomedical Aspects of Botulism, Academic Press, New York, 1981, pp. 233–246.

[3] E. Habermann, F. Dreyer, Clostridial neurotoxins: handling and action at the cellular and molecular level, Top. Microbiolog. Immunol. 129 (1986) 93–179.

[4] G. Schiavo, F. Benfenati, B. Poulain, O. Rossetto, P. Polverino, de Laureto, B.R. Dasgupta, C. Montecucco, Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin, Nature 359 (1992) 832–835.

[5] L.L. Simpson, Molecular pharmacology of botulinum toxin and tetanus toxin, Annu. Rev. Pharmacol. Toxicol. 26 (1986) 427–453.

[6] B.R. DasGupta, The structure of botulinum neurotoxins, in: L.L. Simpson (Ed.), Botulinum Neurotoxin and Tetanus Toxin, Academic Press, New York, 1989, pp. 53–67.

[7] L.L. Simpson, The origin, structure, and pharmacological activity of botulinum toxin.", Pharmacol. Rev. 33 (1981) 155–188.

[8] B. Poulain, U. Weller, T. Binz, H. Neimann, B. De Pavia, O. Dolly, C. Leprince, L. Tauc, Functional roles of the domains of clostridial neurotoxins: the contribution from studies on *Aplysia*, in: B.R. DasGupta (Ed.), Botulinum and Tenanus Neurontoxins: Neurotransmission and Biomedical Aspects, Plenum Press, New York, 1993, pp. 34–360.

[9] J.D. Black, J.O. Dolly, Interaction of $^{125}$I-labeled botulinum neurotoxin with nerve terminals. I. Ultrastructual autoradiographic localization and quantification of distinct membrane acceptors for types A and B on motor nerves, J. Cell Biol. 103 (1986) 521–534.

[10] T.-I. Nishiki, Y. Kamata, Y. Nemoto, A. Omori, T. Ito, M. Takahashi, S. Kozaki, Identification of protein receptor for *Clostridium botulinum* type B neurotoxin in rat brain synaptosomes, J. Biol. Chem. 269 (1994) 10498–10503.

[11] C.C. Shone, P. Hambleton, J. Melling, Inactivation of *Clostridium botulinum* type A neurotoxin by trypsin and purification of two tryptic fragments. Proteolytic cleavage near the COOH-terminus of the heavy subunit destroys toxin-binding activity, Eur. J. Biochem. 151 (1985) 75–82.

[12] G. Schiavo, O. Rossetto, S. Catsicas, P. Poverino, de Laureto, B.R. Dasgupta, F. Benfenati, C. Montecucco, Identification of the nerve terminal targets of botulinum neurotoxin serotypes A, D and E, E.J. Biol. Chem. 268 (1993) 23784–23787.

[13] J.D. Black, J.O. Dolly, Interaction of $^{125}$I-labeled botulinum neurotoxin with nerve terminals. II. Autoradiographic evidence for its uptake into motor nerves by acceptor-mediated endocytosis, J. Cell Biol. 103 (1986) 535–544.

[14] C.C. Shone, P. Hambleton, J. Melling, A 50-kDa fragment from the $NH_2$-terminus of the heavy subunit of *Clostridium botulinum* type A neurotoxin forms channels in lipid vesicles, Eur. J. Biochem. 167 (1987) 175–180.

[15] R.O. Blaustein, W.J. Germann, A. Finkelstein, B.R. Dasgupta, The N-terminal half of the heavy chain of botulinum type A neurotoxin forms channels in planar phospholipid bilayers, FEBS Lett. 226 (1987) 115–120.

[16] M.S. Montal, R. Blewitt, J.M. Tomich, M. Montal, Identification of an ion channel-forming motif in the primary structure of tetanus and botulinum neurotoxins, FEBS Lett. 313 (1992) 12–18.

[17] G. Schiavo, O. Rossetto, F. Benfenati, B. Poulin, C. Montecucco, Tetanus and botulinum neurotoxins are zinc proteases specific for components of the neuroexocytosis Appartus, Annu. N. Y. Acad. Sci. 710 (1994) 65–75.

[18] S. Tonello, S. Morante, O. Rossetto, G. Schiavo, C. Montecucco, Tetanus and botulinum neurotoxins, a novel group of zinc-endopeptisadases, in: K. Suziki, J. Bond (Eds.), Intracelluar Protein Catabolism, Plenum Press, New York, 1996, pp. 251–260.

[19] G. Schiavo, B. Poulain, O. Rossetto, F. Benfenati, L. Tauc, C. Montecucco, Tetanus toxin is a zinc protein and its inhibition of neurotransmitter release and protease activity depends on Zinc, EMBO J. 11 (1992) 3577–3583.

[20] C.C. Shone, C.P. Quinn, R. Wait, B. Hallis, S. Fooks, P. Hambleton, Proteolytic cleavage of synthetic fragments of vesicle-associated membrane protein, isoform-2 by botulinum type B neurotoxin, Eur. J. Biochem. 217 (1993) 965–971.

[21] L.A. Smith, M. Byrne, Vaccines for preventing botulism, in: M. Brin, J. Jankovic, Hallett (Eds.), Scientific and Therapeutic Aspects of Botulinum Toxin, Lippincott, Williams & Wilkens, Philadelphia, 2002, pp. 427–440.

[22] M.A. Clayton, J.M. Clayton, D.R. Brown, J.L. Middlebrook, Protective vaccination with a recombinant fragment of *Clostridium botulinum* neurotoxin serotype A expressed from a synthetic gene in *Escherichia coli*, Infection and Immunity 63 (1995) 2738–2742.

[23] M.P. Byrne, R.W. Titball, J. Holley, L.A. Smith, Fermentation, purification and efficacy of a recombinant vaccine candidate against botulinum neurotoxin type F from *Pichia pastoris*, Prot. Exp. Purif. 18 (2000) 327–337.

[24] L.A. Smith, Development of recombinant vaccines for botulinum neurotoxin, Toxicon. 36 (1998) 1539–1548.

[25] C.L. Hathaway, Toxoid of *Clostridium botulinum* type F: purification and immunogenicity studies, Appl. Environ. Microbiol. 31 (1976) 234–242.

[26] K.J. Potter, M.A. Bevins, E.V. Vassilieva, V.R. Chiruvolu, T. Smith, L.A. Smith, M.M. Meagher, Production and purification of the heavy-chain fragment C of botulinum neurotoxin, serotype

B, expressed in the methylotrophic yeast *Pichia pastoris*, Prot. Exp. Purif. 13 (1998) 357–365.

[27] K.J. Potter, W. Zhang, L.A Smith, M.M. Meagher, Production and purification of the heavy chain fragment C of botulinum neurotoxin, serotype A, expressed in the methylotrophic yeast *Pichia pastoris*, Prot. Exp. Purif. 19 (2000) 393–402.

[28] T. Kubota, T. Watanabe, N. Yokosawa, K. Tsuzuki, T. Indoh, K. Moriishi, K. Sanda, Y. Maki, K. Inoue, N. Fujii, Epitope regions in the heavy chain of *Clostridium botulinum* type E neurotoxin recognized by monoclonal antibodies, Appl. Environ. Microbiol. 63 (1997) 1214–1218.

[29] V. Chiruvolu, J.M. Cregg, M.M. Meagher, Recombinant protein expression in an alcohol oxidase-defective strain of *Pichia pastoris* in feed-batch fermentations, Enzyme Microbiol. Technol. 21 (1997) 277–283.

[30] F. Sherman, R.P. Moerschell, S. Tsunasawa, R. Sternglanz, in: K. Imahori, F. Sakiyama (Eds.), Methods in Protein Sequence analysis, Plenum, New York, 1993, pp. 173–181.